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Molecular recognition of nucleic acids: Coralyne binds strongly to poly(A)

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Abstract **The small molecule coralyne was found to bind preferentially and strongly to single-stranded poly(A) with an apparent association constant (K_a) of $(1.8 \pm 0.3) \times 10^6\ M^{-1}$. Binding of coralyne to poly(A) is predominantly enthalpically driven with a stoichiometry of one coralyne per four adenine bases. Poly(A) forms a coralyne dependent secondary structure with a melting temperature of 60 °C, for the conditions of our study.**

Keywords: DNA; RNA; Dialysis; Binding; Single-stranded poly(A); Secondary structure

1. Introduction

The essential role played by RNA in normal biological processes and in the progression in many diseases has led to a growing interest in exploiting RNA as a target for the design of new classes of therapeutics [1–6]. Ribosomal RNA has long been known to be the receptor for antibiotics, and antisense oligonucleotides are being used to downregulate gene expression [3,5]. RNA viruses, such as AIDS or hepatitis C virus, are targets that urgently need new types of antiviral drugs [3,6]. In non-infectious diseases, the opportunities for targeting RNA are also abundant but unexplored. New drugs could potentially be designed to bind to unique structural regions in mRNA and thereby regulate gene expression. For example, virtually all mRNAs have a number of adenylate residues at their 3′-end, the poly(A) tail. The long poly(A) tail is an important determinant of mRNA stability and maturation, and is essential for the initiation of translation [7,8]. Poly(A) polymerase (PAP) catalyzes 3′-end poly(A) synthesis, participates in an endonucleolytic cleavage step, and is one key factor in the polyadenylation of the 3′-end of mRNA. Neo-PAP, a recently identified human PAP, is significantly over expressed in human cancer cells in comparison to its expression in normal or virally transformed cells [9,10], and may represent a tumor-specific target. Drugs capable of recognizing and binding to the single-stranded A-rich regions of mRNA might interfere with the full processing of mRNA by PAP and would represent a new type of potential therapeutic agent.

Despite many studies that probed the interactions of drugs with nucleic acids, little is known about drug recognition of single-stranded, A-rich RNAs. Very few drugs, in fact, seem to bind to single-stranded nucleic acids at all [11]. Coralyne (Fig. 1, top panel) has been shown to exhibit significant antitumor activity against both P388 and L1210 leukemias in mice [12]. Past studies have shown that coralyne intercalates both duplex and triplex DNA structures, but with higher affinity to triplex [13,14]. Our previous competition dialysis assay using 13 nucleic acid structural forms revealed the preferential binding to poly(dA):poly(dT)$_2$ triplex DNA, and identified unusually strong binding to poly(dA) [15]. Recent work by Polak and Hud and Persil et al. [16,17] has shown the comparable affinities of coralyne for poly(dA) and triplex DNA, and demonstrated that coralyne could disproportionate duplex poly(dA):poly(dT) into a triplex form and a poly(dA)–coralyne complex. In our search for promising compounds for selectively targeting of poly(A) tail in the 3′-end of mRNA, an expanded 19-sample competition dialysis method (which is now firmly established as an important tool for rapidly screening drug–nucleic interaction [15,18–20]) was explored. We report here that coralyne can recognize and bind strongly to single-stranded poly(A), inducing the formation of a self-structure of poly(A) with a melting temperature of 60 °C, for the conditions of our study.

2. Materials and methods

2.1. Materials

DNA samples from *Clostridium perfringens*, *Micrococcus lysodeikticus* and calf thymus were purchased from Sigma (St. Louis, MO, USA) and were sonicated, phenol-extracted and purified before use. Poly(dA), poly(dT), poly(dC), poly(U), poly(dA–dT), poly(dA):poly(dT), poly(dG–dC) were purchased from Pharmacia Biotech (Piscataway, NJ, USA). Poly(A) was purchased from Sigma. 5′-$AG_3(T_2AG_3)_3$, 5′-$T_4G_{10}T_4$, and 5′-$T_2G_{20}T_2$ were purchased from Research Genetics (SW Huntsville, AL, USA). They were used without further purification. Sample preparation was done as previously described [15,18–20]. Concentrations of nucleic acids samples were determined by UV absorbance measurements using the extinction coefficients and absorbance maxima listed in Table S1. Coralyne was obtained from Aldrich (Milwaukee, WI, USA), and the concentration was determined by visible absorbance measurement using extinction coefficient $\varepsilon_{420} = 14\,500\ M^{-1}\,cm^{-1}$. A buffer consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 19 mM NaCl (pH 7.0) was use for all experiments except a BPES buffer containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0) in competition dialysis assay.

2.2. Competition dialysis assay

Full details of the competition dialysis assay may be found in [15,18]. A brief description follows. In the competition dialysis experiment, 19 different nucleic acid samples (at identical concentration of 75 μM) were dialyzed against test drug solution (at 1 μM concentration).

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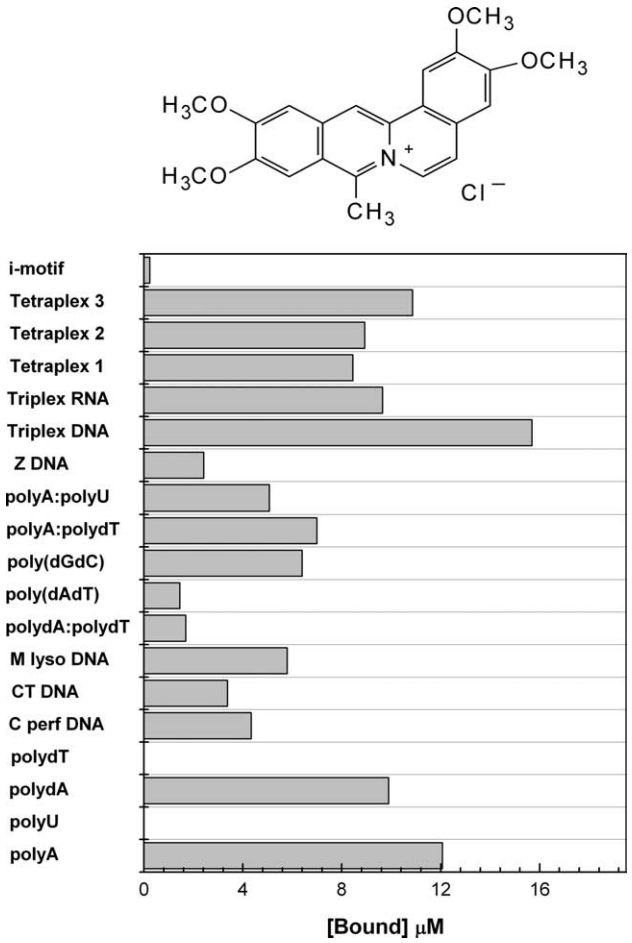

Fig. 1. Top: Structure of coralyne. Bottom: results of competition dialysis experiment. The concentration of coralyne bound to each nucleic acid sample is shown as a bar graph.

Following equilibration, the free drug concentration (C_f) was determined spectrophotometrically using an aliquot of the dialysate solution, and the total concentration of coralyne (C_t) with each dialysis unit was then determined by absorbance measurements. The amount of bound drug was determined by difference, $C_b = C_t - C_f$. Data were plotted as a bar graph using Origin Software.

2.3. Continuous variations analysis (Job plot)

Full experimental details are done as previously described [16]. Stock solution of coralyne and poly(A) (in unit of nucleotide base/4) were prepared at equivalent concentration of 50 μM, respectively. Portions of each solution were mixed at the appropriate volumetric ratios and, after equilibration, the absorbance at 412 and 435 nm were measured. The ration of coralyne absorption at 412 versus 435 nm was plotted as a function of relative concentrations of coralyne to poly(A) (in units of nucleotide base/4).

2.4. Circular dichroism (CD)

CD spectra were acquired on a Jasco 810 CD spectrometer equipped with a Julabo temperature control unit. Spectra of poly(A) (60 μM) were acquired at 20 °C in the absence and presence of 9 μM coralyne using a 10 mm path length cell. CD melting profiles were generated by increasing the sample (40 μM poly(A) and 8 μM coralyne) temperature at a rate of 1 °C/min, with the absorbance being continuously monitored at 320 or 263 nm.

2.5. Fluorescence titration experiments

Full experimental details and analysis are done as previously described [21]. Fluorescence titration experiment was carried out at 20 °C on a Jasco 6500 spectrafluorometer. A fixed concentration (1 μM) of coralyne was titrated with increasing concentration of poly(A) while monitoring fluorescence emission intensity. Binding curves were analyzed by an iterative nonlinear least-squares fitting method (FitAll, Toronto).

2.6. Isothermal titration calorimetry

ITC experiments were carried out with an isothermal microtitration calorimeter (ITC Model 4200, Calorimetric Sciences Corp. Provo, UT). Titrations were carried out at 20 °C. Standard protocols for sample preparation and injection were followed as previously described [22]. The poly(A) concentration in ITC experiments was 1 mM (nucleotide). Serial injections (3 μL) of a stock coralyne solution (1 mM) were added at 300 s time intervals to the poly(A) reaction solution. Control experiments were carried out to calculate the heats of dilution for drug titrated into buffer. The net enthalpy for each drug–DNA interaction was determined by subtracting the heats of dilution for the buffer from the drug–DNA titration curves. At least three titration experiments were performed for each poly(A) molecule in the set. The experimental error of the heat signal was not more than 10%.

3. Results and discussion

Fig. 1 shows the result from the competition dialysis assay. 19 different nucleic acid samples (at identical concentrations of 75 μM) are dialyzed against 1 μM coralyne solution. Table S1 (Supporting Information) described each nucleic acid form. Following equilibration, more drug accumulates in the dialysis tube containing the structural form of highest binding affinity. Data are shown as a bar graph, in which the concentration of coralyne bound to each nucleic acid sample is plotted. The striking result to emerge here in the expanded, 19-samples competition dialysis assay is the pronounced binding of coralyne to single-stranded poly(A). Triplex DNA binds the most coralyne, with poly(A) representing the next most preferred form. Poly(dA), tetraplex DNA and an RNA triplex (poly(A):-poly(U)$_2$) also bind coralyne well, while other nucleic acid structures show little or no binding. We note that the RNA samples described here were limited to the poly(A) and poly(U). There exists a variety of RNA structural forms, and coralyne may bind with affinity and specificity to certain RNA structures through shape specific/or sequence specific. It is certainly desirable to investigate the interaction of coralyne with additional type of RNA structures, and we intend to continue to develop the competition dialysis assay to include more structural forms of interest along with a range of ionic conditions.

The binding of coralyne to poly(A) was further examined by fluorescence intensity measurements in a buffer containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 19 mM NaCl. Coralyne is strongly quenched upon binding and an apparent association constant (K_a) of $(1.8 \pm 0.3) \times 10^6$ M^{-1} was determined (shown in Fig. S1). A binding free energy of -8.4 kcal mol^{-1} was determined using the standard equation $\Delta G = -RT\ln K$. The enthalpy change associated with the formation of coralyne–poly(A) complex was measured directly by isothermal titration calorimetry (shown in Fig. 2) using the "model-free ITC" protocol [22]. The heat of dilution was subtracted from the enthalpy of reaction to give the enthalpy of binding of (-8.3 ± 0.6) kcal mol^{-1}. The binding free energy, coupled with the binding enthalpy derived from the ITC data, allowed us to calculate the corresponding entropic contributions to binding. The small entropy term, $T\Delta S = 0.1$ kcal mol^{-1}, indicates that binding to the poly(A) is predominantly enthalpically driven.

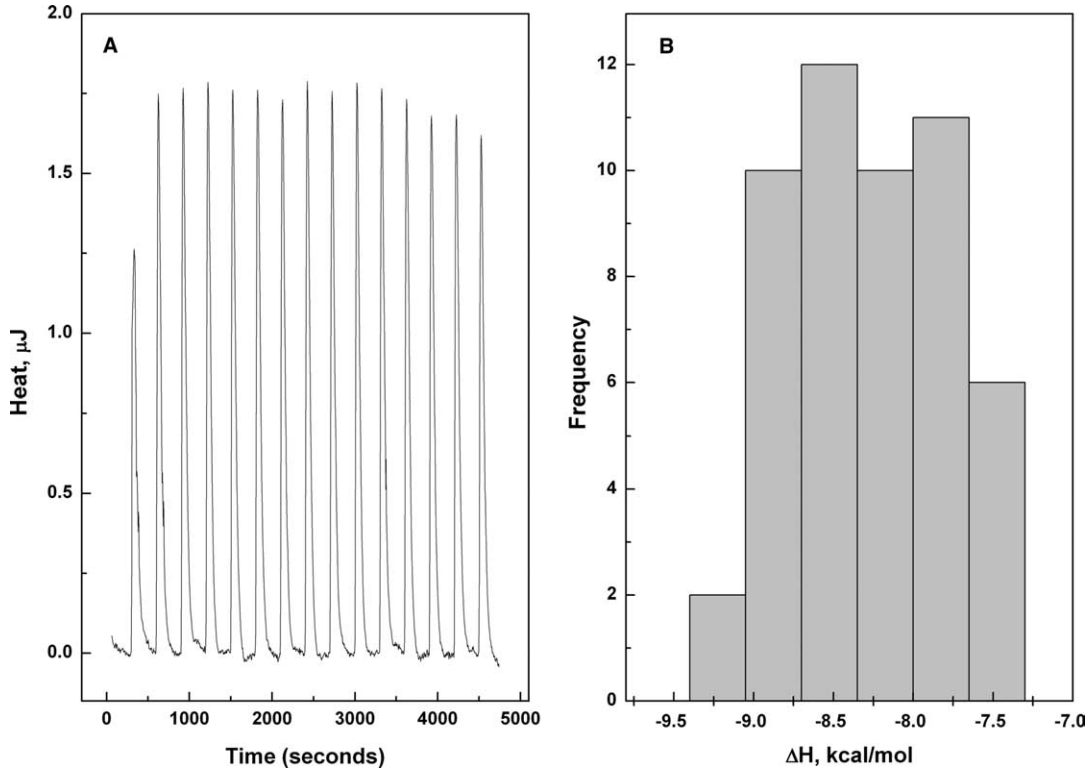

Fig. 2. (A) Representative primary data from an ITC experiment. Each peak shows the heat produced by a serial injection of an aliquot of coralyne solution(3 μl of 1 mM) into the poly(A) solution (1 mM nucleotide). (B) Distribution of poly(A)–coralyne binding enthalpy value. The distribution was obtained from three independent titrations with 20 ligand injections in each ITC experiment.

CD spectra shown in Fig. 3A demonstrate that the conformation of poly(A) undergoes a dramatic change in the presence of coralyne. The decrease in intensity of CD bands at ~250 and 260 nm and a new small negative band around 290 nm all implies the alteration of poly(A) structure upon addition of coralyne. The appearance of positive bands between 320 and 340 nm, and the small peak ~435 nm indicate the binding of coralyne with the chiral environment of poly(A). Poly(A) is known to form a duplex with parallel strands and protonated adenine bases under acidic conditions [23]. The Hud laboratory's recent studies of the remarkable ability of coralyne to disproportionate poly(dA):poly(dT) into a triplex and a coralyne–poly(dA) complex have revealed that poly(dA) could form a self-structure [16,17]. To determined if poly(A) forms a self-structure with coralyne, we use temperature dependent CD spectropolarimetry at various wavelength to investigate the melting of the complex. Fig. 3B shows the CD melting profile of poly(A) containing coralyne. With poly(A) or only coralyne, no CD melting profiles were observed. However, in the presence of coralyne, the CD melting profiles at either 274 or 320 nm reveal a melting transition around 60 °C, suggesting that coralyne binding induces formation of self-structure of poly(A).

The poly(A) self-structure formation in the presence of coralyne was further investigated by UV–Vis spectrophotometry. Fig. 4A shows the absorption spectrum of coralyne alone and in the presence of poly(A). In the region of 380–480 nm, the spectrum of free coralyne has a maximum around 420 nm. Upon addition of poly(A) to coralyne, the coralyne undergoes dramatic shifts with two local maximum at 412 and 435 nm. To establish the stoichiometries of the complex formed by coralyne and poly(A), we use the method described by Polak [16] to construct a continuous fraction analysis (Job plot). For this analysis the ration of coralyne absorption at 412 versus 435 nm was plotted as a function of relative concentrations of coralyne to poly(A) (in units of nucleotide base/4), with the sum total concentration of coralyne and adenine nucleotide base held constant at 50 μM. Fig. 4B reveals an inflection point at 0.5 *R* ratios, establishing the formation of one coralyne per four adenine bases. In the aggregate, the UV–Vis absorption profile and Job plot reveal that coralyne intercalates the poly(A) self-structure and the poly(A) self-structure obeys the nearest neighbor exclusion principle with intercalation between every other stacked base pair. The large, negative binding enthalpy for the interaction (-8.3 kcal mol^{-1}) is typical of intercalative binding [24–26]. Poly(dA) was found to form a self-structure with base pairing in the presence of coralyne which was supported by the cooperative melting transition and Job plot analysis [16]. Our results show that changes in CD spectra, UV–Vis absorption profiles and the behavior observed in Job plots are similar for poly(dA) and poly(A), suggesting similar structural changes. Comparison of the self-structure formed by these two polymer in the presence of coralyne suggests that poly(A) could possibly form the same type of base pairing. Poly(A) was reported to form a duplex with parallel strands and protonated adenine bases under acidic conditions [23]. Recent work by Persil [17] has demonstrated that coralyne promotes the formation of an antiparallel homo-adenine duplex at neutral pH, and the homo-adenine–coralyne duplex is significantly different from the low-pH poly(A) duplex. The similarity for poly(dA)–coralyne and poly(A)–coralyne complex suggested that a similar antiparallel

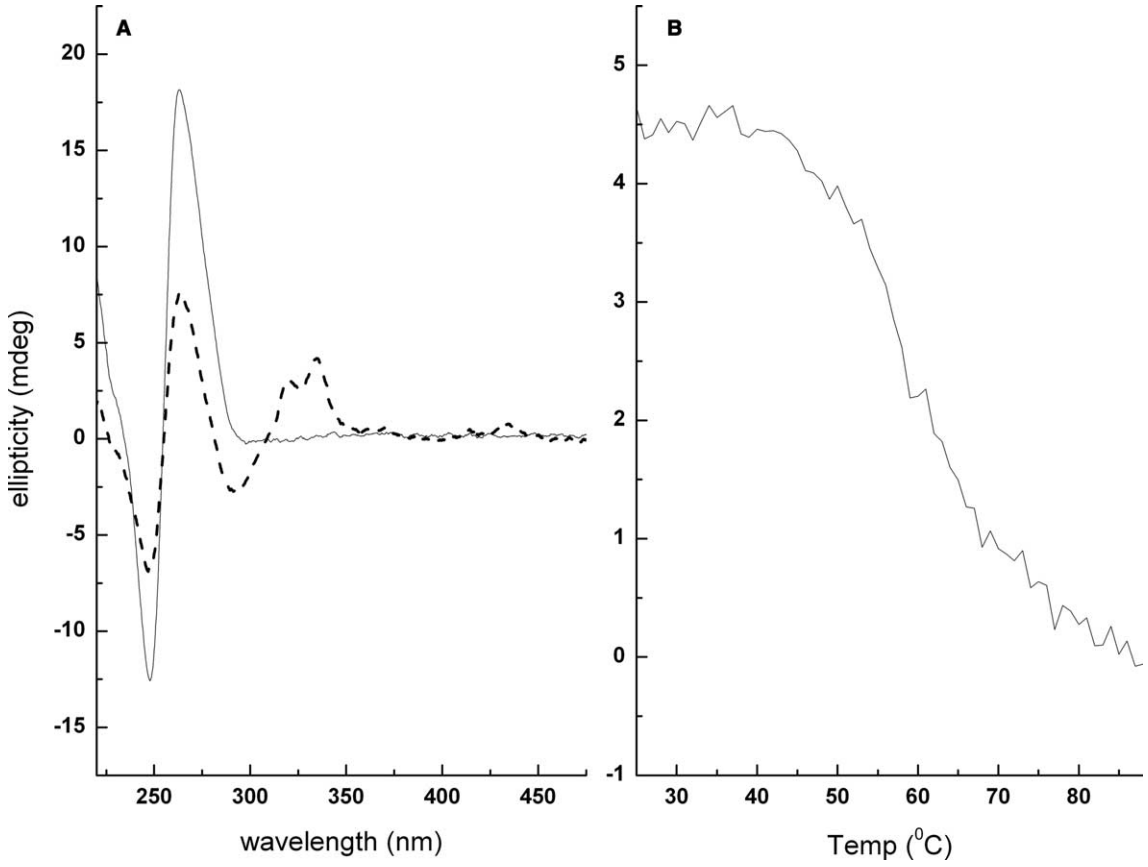

Fig. 3. (A) CD spectra of poly(A) (60 μM) acquired at 20 °C in the absence (solid line) and presence (dashed line) of 9 μM coralyne. (B) CD melting curve of solution containing 40 μM poly(A) and 8 μM coralyne.

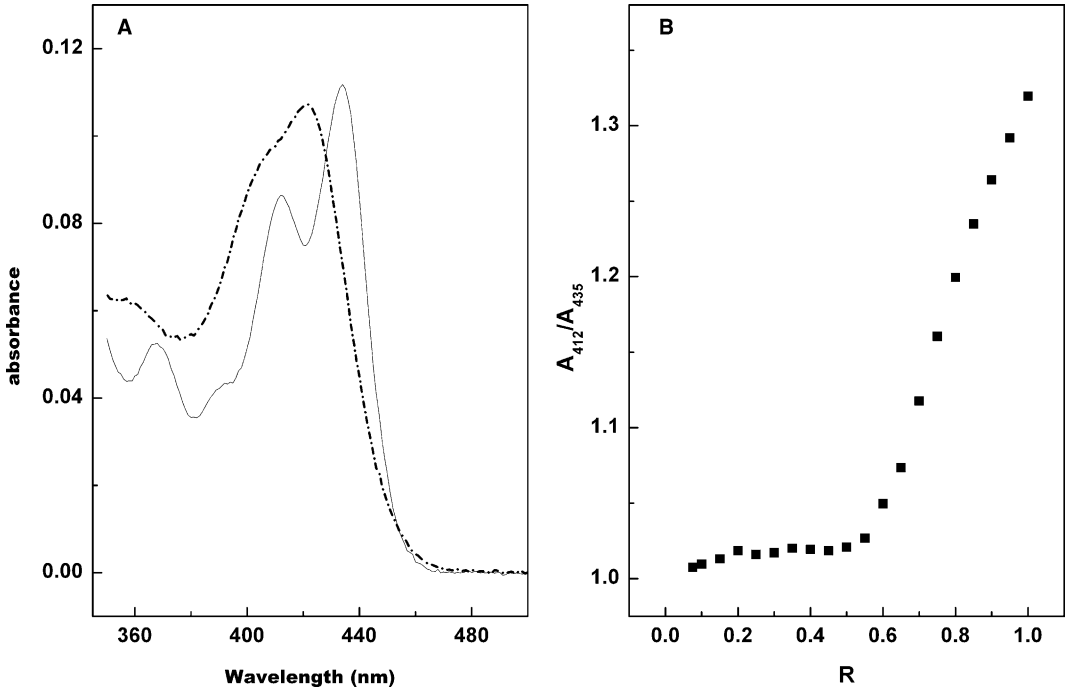

Fig. 4. (A) UV–Vis profiles of 7.5 μM coralyne in the presence of poly(A) at 150 μM adenine base. Dashed line is coralyne alone and solid line is with poly(A). (B) Job plot of poly(A) with coralyne. Sample condition was 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 19 mM NaCl.

strand arrangement is also likely to exist in the poly(A)–coralyne complex; however, a parallel strand arrangement cannot be ruled out and additional structural studies are required to clarify this point.

Small molecules that bind selectively and with high affinity to single stranded nucleic acid are rare [11]. Coralyne is unique in its ability to selectively recognize poly(dA) and poly(A). While the exact mode of its binding is not yet clear, that fact that it does bind selectively to these polynucleotides is unambiguous. Further studies will be required to elucidate the structure of the coralyne–polynucleotide complex, but these are beyond the scope of this brief communication. Coralyne represents a promising lead compound for the design of new compounds that might recognize single-stranded nucleic acids, and which may represent a new avenue for the development of therapeutic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.07.091.

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